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(54) Title: COMPOSITIONS AND METHODS FOR INDUCING APOPTOSIS IN E6-EXPRESSING CELLS (57) Abstract Methods, pharmaceutical compositions and kits are provided for inducing programmed cell death in cells expressing the E6 oncogene. The methods and compositions are particularly suited for treatment of cancers involving infections with E6-expressing virus, such as human papilloma virus (HPV). The methods and compositions utilize the p53 homolog, p73. Unlike p53, p73 is not targeted by the E6 oncoprotein for ubiquitin-mediated degradation, and so provides a viable alternative to p53 therapy for treatment of E6-expressing cancers.		

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**COMPOSITIONS AND METHODS FOR INDUCING
APOPTOSIS IN E6-EXPRESSING CELLS**

This application claims priority to U.S. Provisional Application 60/090,526, filed June 24, 1998, the entirety of which is incorporated by reference herein.

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FIELD OF THE INVENTION

This invention relates to the field of methods of treatment of cancer. In particular, this invention provides a method of treatment of cancers associated with human papillomavirus infection or other tumors in which the E6 oncogene is expressed, and a pharmaceutical preparation and kit to practice the method.

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BACKGROUND OF THE INVENTION

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Various scientific and scholarly articles and patents are referred to in brackets throughout the specification. These articles and patents are incorporated by reference herein to describe the state of the art to which this invention pertains.

20

Infection with human papillomavirus (HPV) is a major risk factor for the development of squamous cell carcinoma of the cervix. The E6-oncoprotein encoded by HPV has been shown to target the tumor suppressor protein p53 for degradation via ubiquitin conjugation and subsequent proteolysis (Scheffner et al., 1990, Cell 63: 1129-1136). HPV-E6-expressing cancer cells are resistant to the tumor suppressive effects of exogenous wild-type

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p53 delivered by an adenovirus (Ad) vector (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229).

Several approaches have been proposed to control the growth of HPV E6-expressing cancer cells. These include the use of p21-expressing adenovirus to bypass the p53-degradation step (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229), the use of bovine papillomavirus E2 gene to reactivate endogenous p53 (Hwang et al., 1996, Oncogene 12:795-803), the use of hypoxic conditions to suppress p53 degradation (Kim et al., 1997, Cancer Res. 57:4200-4204), the use of alternatively spliced E6 to compete with normally spliced E6 (Pim et al., 1997, Oncogene 15: 257-264), the use of antisense strategies to lower E6 expression (Hamada et al., 1996, Gyn. Onc. 63:219-227; Beer-Romero et al., 1997, Oncogene 14: 595-602), and the generation of p53 mutants resistant to ubiquitin-directed degradation (Crook et al., 1996, Virology, 217:285-292). In the last mentioned approach, it was found that, although lysine mutants of the C-terminus of p53 did resist E6-mediated degradation *in vitro*, the effect was not observed in intact cells, where the lysine mutant was efficiently targeted for degradation (Crook et al., 1996, Virology, 217:285-292). Some tumor-derived mutants of p53 may also be resistant to E6-dependent proteolysis *in vitro* (Medcalf and Milner, 1993, Oncogene 8:2847-2851). p21-expressing adenovirus (Ad-p21) inhibits the growth of E6-over-expressing cells, although the primary effect of p21 over-expression is a growth arrest associated with a large cell phenotype and little, if any, apoptosis (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229; Meng et al., 1998, Clin. Cancer Res. 4: 251-259). Thus, none of the aforementioned approaches has been

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particularly successful in controlling the growth of E6-over-expressing cells by induction of programmed cell death.

Alternative strategies for the suppression of growth of E6-expressing cancer cells are of great utility. Such alternative strategies would ideally induce apoptosis of the E6-over-expressing cells, as well as inhibit cell proliferation.

10 SUMMARY OF THE INVENTION

Therapy based on the p53 tumor suppressor is unavailable for cancers associated with expression of the E6 oncogene because the E6 protein targets p53 for degradation by ubiquitin-mediated proteolysis. It has been discovered in accordance with the present invention that the p53 homolog, p73, is not targeted for degradation by E6 and, moreover, is a potent inhibitor of cancer colony growth and inducer of apoptosis, even in cells that over-express E6. Thus, p73 is a superior tumor suppressor protein for treatment of cancers in which the E6 oncogene is expressed, such as those associated with HPV infection.

According to one aspect of the present invention, method is provided for inducing apoptosis in an E6-expressing cell. The method comprises administering to the cell an amount of p73 protein effective to induce the apoptosis. In one embodiment, the p53 protein is administered as a DNA construct comprising an expressible sequence that encodes the protein. Preferably, the DNA construct is operably inserted into a viral vector for transforming cells.

The method is typically utilized for arresting growth of cancerous cells, particularly cancers

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associated with infection with E6-expressing viruses, such as HPV. In one embodiment, the cell is a cultured cell. In another embodiment, the cell is obtained from the body of a living organism, the administering is performed *ex vivo*, and the cell is returned to the living organism. In still another embodiment, the cell is disposed within a living organism and the administering is performed *in vivo*.

The p73 protein utilized in the method is preferred to be p73 α or p73 β , most preferably the latter. In a preferred embodiment, the protein comprises a sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2. If a DNA construct is used, the DNA construct preferably comprises more than 50 nucleotides of SEQ ID NO:3.

According to another aspect of the invention, an apoptotic, E6-expressing transgenic cell is provided, which comprises a heterologous, expressible DNA construct encoding p73. In one embodiment, the cell is obtained from a cultured cell line. In another embodiment, the cell is a primary cell obtained from a living organism. In yet another embodiment, it is disposed within a living organism.

According to another aspect of the invention, a pharmaceutical preparation for treatment of cancers associated with E6 over-expression is provided. In one embodiment, the pharmaceutical preparation comprises a p73 protein associated with a delivery vehicle for delivering proteins to cancer cells. In another embodiment, the preparation comprises an expressible DNA construct encoding p73, associated with a delivery vehicle for delivering DNA to cancer cells. The

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pharmaceutical preparation also may comprise at least one additional active ingredient for treatment of cancer.

According to another aspect of the invention, a kit is provided that contains the pharmaceutical preparation and other optional components. For instance, in a preferred embodiment, the kit may include a second pharmaceutical agent useful for treating cancer.

Other features and advantages of the present invention will be better understood by reference to the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Ad-E6 infection leads to degradation of both wild-type and mutant p53 in human cancer cells. The human brain (U373, H80; lanes 1-4), breast (SKBr3; lanes 5,6), lung (H460; lanes 7,8), or colon (HCT116, SW480; lanes 9-12) cancer cell lines were infected using Ad-LacZ or Ad-E6 (as indicated). Immunoblotting for p53 expression (upper panels) or pRb expression (lower panels) was carried out as described in Example 1. pRb expression is presented to document equivalent loading between lysates derived from Ad-LacZ and Ad-E6 infected cells. For cell lines that express mutant p53, the following mutations have been previously reported: U373 cell line: R273H (Kaghad et al., 1997, Cell 90: 809-819); SW480 cell line: R273H, P309S (Kaghad et al., 1997, Cell 90: 809-819); SKBr3 cell line: R175H (Kovach et al., 1991, J. Natl. Cancer Inst., 83:1004-1009); H80 cell line (also known as U-373 MG): R273H (Gomez-Manzano et al., 1996, Cancer Res. 56:694-699).

Figure 2. p73, unlike p53, is not specifically targeted for degradation in Ad-E6 infected cancer cells. SW480 cells were transfected by p73 α (lanes 1,2), p73 α m

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(lanes 3,4), p73 β (lanes 5,6), or p73 β m (lanes 7,8). At six hours following transfection, cells were infected by either Ad-LacZ or Ad-E6 (as indicated). At 20 hrs. following infection, expression of p73 α (upper left) or p73 β (upper right) was detected by immunoblotting using anti-HA antibody and for p53 (lower panels) expression using anti-p53 antibody, as described in Example 1. The band just above p73 α is a non-specific anti-HA cross-reactive band.

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DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specifications and claims.

With reference to nucleic acid molecules, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it

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exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to proteins or polypeptides, the term "isolated protein (or polypeptide)" or "isolated and purified protein (or polypeptide)" is sometimes used
5 herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other
10 proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid,
15 oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods,
20 agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus
25 define the differences. For purposes of this invention, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program are the parameters
30 intended to be used herein to compare sequence identity and similarity. Alternatively, standard BLAST query parameters, utilized by public databases such as GenBank, are utilized herein.

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The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the protein (i.e. the structure, thermostability characteristics and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of

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the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

Transcriptional and translational control sequences, sometimes referred to herein as "expression control" sequences or elements, or "expression regulating" sequences or elements, are DNA regulatory elements such as promoters, enhancers, ribosome binding sites, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell. The term "expression" is intended to include transcription of DNA and translation of the mRNA transcript.

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a

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selectable phenotype such as antibiotic resistance on a transformed cell.

The term "operably linked" means that the regulatory sequences necessary for expression of a particular coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other regulatory elements (e.g., enhancers or translation regulatory sequences) in an expression vector.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself

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is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do
5 not give rise to a heterologous region of DNA as defined herein.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or
10 may not be integrated (covalently linked) into the genome of the cell. For example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become
15 integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing
20 the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

"Killing", "programmed cell death" and
25 "apoptosis" are used interchangeably in this text to describe a series of cellular events that culminates in the death of the target cell. Apoptosis is a characteristic morphological change in which the cell and its nucleus shrink, condense and fragment. Frequently
30 accompanying this morphological change are the activation of intracellular proteases and nucleases that lead to, for example, cell nucleus involution and nuclear DNA fragmentation.

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II. Description

Human papillomavirus (HPV) is the major cause of cervical cancer worldwide. HPV-E6 protein targets the p53 tumor suppressor protein for degradation by ubiquitin-mediated proteolysis, making such cancers resistant to p53-mediated therapy.

In accordance with the present invention, two discoveries have been made that have significant implications and suggest novel strategies for cancer therapy. The first discovery is that HPV-E6 targets both endogenous wild-type and mutant p53 for degradation (Fig. 1). Possibly because p53 mutations are rare in cervical cancer (Busby-Earle et al., 1994, Br. J. Cancer 69: 732-737) the hypothesis that HPV-E6 could target endogenous mutant p53 for degradation has not been previously directly tested. While several studies have reported low levels of p53 expression and an inverse correlation between the presence of HPV and p53 expression (Scheffner et al., 1991, Proc. Natl. Acad. Sci. USA 88: 5523-5527; Srivastava et al., 1992, Carcinogenesis 13: 1273-1275; Baret et al., 1996, Eur. J. Gyn. Onc. 17: 283-285; Hachisuga et al., 1996, Pathology 28: 28-31), there is apparently no such correlation between p53 mutation and HPV (Busby-Earle et al., 1994, Br. J. Cancer 69: 732-737; Kim and Kim, 1995, Yonsei Med. J. 36:412-425). While the discovery that HPV-E6 also targets mutant p53 for degradation provides no insight into how the rare p53 mutations may contribute to HPV-associated cervical cancer, it is consistent with the known inverse correlation between p53 expression and the presence of HPV in high risk cervical cancer.

The second and more significant discovery is that the p53 homolog, p73, is not targeted for

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degradation by the E6 oncoprotein. Furthermore, as described in greater detail below and in Example 1, p73 is a potent inducer of apoptosis and is an effective inhibitor of cancer cell growth. For such HPV

5 E6-expressing cancers where p53 is degraded and fails to control growth, p73 is an excellent substitute for p53 in gene replacement because of its resistance to E6-mediated proteolysis. Other differences of effect of viral oncoproteins have been noted (Marin et al., 1998, Mol.

10 Cell. Biol. 18:6316-6324; Steengenga et al., 1999, Mol. Cell. Biol. 19:3885-3894; Dobbstein and Roth, 1998, J. Gen Virol. 79:3079-3083; Roth et al., 1998, J. Virol. 72:8510-8516; Reichelt et al., 1999, Arch. Virol. 144:621-626). It is noteworthy that, even though p73 has

15 the potential to interact with p53 in a yeast two-hybrid analysis (Kaghad et al., 1997, Cell 90: 809-819), the expressed p73 is not subject to E6-dependent proteolysis under conditions where high levels of endogenous mutant p53 are degraded (Fig. 2).

20 Provided with this invention are methods, pharmaceutical preparations and kits that utilize p73 for arresting the growth of E6-expressing cells, particularly HPV-infected cancer cells. The treatment of the target cells may be *in vivo*, within the patient; or *ex vivo*,

25 removed from the patient, treated, and reintroduced into the patient. It is contemplated that the methods, pharmaceutical preparations and kits of the invention can be used alone or in conjunction with chemotherapy or radiation therapy to treat cancers *in vivo*.

30 Additionally, the methods, pharmaceutical preparations and kit of the invention can be used for experimental purposes *in vitro* with standard cell cultures.

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As mentioned, the treatment of cancers associated with the over-expression of E6 protein is of particular interest. Several circumstances may result in mammalian cells that over-express E6 protein. Commonly, this nature of cell results from an infection with human papillomavirus (HPV) wherein the E6-oncogene encoded by the virus is expressed in the cell. HPV infection is well-known to result in cancers of the uterine cervix. In addition to anogential cancer, HPV infection may also result in esophageal squamous cell cancer, laryngeal papilloma, bronchiolo-alveolar carcinoma, penile carcinoma and bladder carcinoma, among others. Additionally, E6-over-expression may also result from a mutation in the mammalian cell genome such that the endogenous E6 gene is over-expressed. All mammalian cells that over-express the E6 protein, regardless of the origin of the phenotype, are contemplated for treatment with the method of the invention.

The following description set forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1999) (hereinafter "Ausubel et al.") are used.

Any p73 variant and the nucleic acid sequence encoding it are considered suitable for use in the present invention. In this regard, it should be noted that the two major splice variants of p73, p73 α and p73 β ,

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both have been found resistant to E6-mediated degradation (see Example 1), though p73 β appears to be somewhat more effective in this regard and is preferred for the practice of the present invention.

5 The amino acid sequence of p73 protein on which to base the nucleic acid construct is ideally from the gene that is endogenous to the species which is being treated. In a preferred embodiment, *Homo sapiens* is being treated and the nucleic acid construct encodes SEQ
10 ID NO:1 or SEQ ID NO:2. In a most preferred embodiment, the nucleic acid sequence is SEQ ID NO:3. Other variants of p73 protein also exist in *Homo sapiens* and the sequences of these variants are also contemplated for use with the invention (DeLaurenzi et al., 1999, Cell Death
15 Differ. 6:389-390 incorporated by reference herein; Genbank Accession No. Y11416 incorporated by reference herein).

 The availability of amino acid sequence information, such as the full length sequence in SEQ ID
20 NO:1 and SEQ ID NO:2 enables the preparation of a synthetic gene that can be used to synthesize the *Homo sapiens* p73 protein in standard *in vivo* expression systems or to make viral vectors expressing the p73 protein. The sequence encoding *Homo sapiens* p73 from
25 isolated native nucleic acid molecules such as SEQ ID NO:3 can be utilized. The amino acid and nucleic acid sequences found in Genbank Accession Nos. AF138873, Y11419 and AF043641 can be used to prepare the p73 protein endogenous to *Mus musculus*, *Chlorocebus aethiops*
30 and *Barbus barbus*, respectively. Alternately, an isolated nucleic acid that encodes the amino acid sequence of the invention can be prepared by oligonucleotide synthesis. Codon usage tables can be

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used to design a synthetic sequence that encodes the protein of the invention. In a preferred embodiment, the codon usage table has been derived from the organism in which the synthetic nucleic acid will be expressed. For example, the codon usage for *E. coli* would be used to design an expression DNA construct to produce the *Homo sapiens* p73 in *E. coli*.

Synthetic oligonucleotides may be prepared by the phosphoramidite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant oligonucleotide may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC).

Nucleic acid molecules encoding p73 also may be isolated from appropriate species using methods well known in the art. Native nucleic acid sequences may be isolated by screening mammalian or other cDNA or genomic libraries with oligonucleotides preferably designed to match the *Homo sapiens* coding sequence of p73 (SEQ ID NO:3). Several other p73 amino acid sequences are now known: *Mus musculus*, Genbank Accession No. AF138873; *Chlorocebus aethiops* (Green Monkey), Genbank Accession No. Y11419; and *Barbus barbus*, Genbank Accession No. AF043641; each of these sequences is incorporated by reference herein. Oligonucleotides designed to match any of these sequences or to match regions of high homology between these sequences may also be used to screen for mammalian p73-encoding nucleotides. In positions of degeneracy where more than one nucleic acid residue could be used to encode the appropriate amino acid residue, all the appropriate nucleic acids residues may be incorporated to create a mixed oligonucleotide population, or a neutral base such as inosine may be

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used. The strategy of oligonucleotide design is well known in the art (see also Sambrook et al., Molecular Cloning, 1989, Cold Spring Harbor Press, Cold Spring Harbor NY). Alternatively, PCR (polymerase chain
5 reaction) primers may be designed by the above method to match a known coding sequence of p73, and these primers used to amplify the native nucleic acids from isolated mammalian cDNA or genomic DNA.

Nucleic acids having the appropriate sequence
10 homology with a *Homo sapiens* p73 synthetic nucleic acid molecule may be identified by using hybridization and washing conditions of appropriate stringency. One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid
15 molecules of a specified sequence homology (Sambrook et al., 1989, *supra*):

$$T_m = 81.5^{\circ}\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41(\% \text{G+C}) - 0.63 (\% \text{formamide}) - 600/\text{\#bp in duplex}$$

20 As an illustration of the above formula, using $[\text{Na}^+] = [0.368]$ and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C . The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than
25 about 75% sequence identity would be observed using a hybridization temperature of 42°C .

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid
30 cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a suitable *E. coli* host cell.

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P73 protein can be produced by using *in vitro* expression methods known in the art. For example, part or all of a DNA molecule, such as a DNA encoding the amino acid sequence SEQ ID NO:1 or SEQ ID NO:2, may be
5 inserted into a plasmid vector adapted for expression in a bacterial cell, such as *E. coli*, or a eukaryotic cell, such as *Saccharomyces cerevisiae* or other yeast. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the
10 recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by
15 immunological interaction with antibodies that bind specifically to the recombinant protein or fusion proteins such as His tags. Such methods are commonly used by skilled practitioners.

The method of the invention for treating
20 mammalian cells that over-express E6 comprises administering a therapeutically effective amount of p73 protein to the target cells. The administration of the p73 protein can be accomplished via several methods, including the exposing the target cell, i.e., the E6-
25 over-expressing cell, to p73 protein, or exposing the target cell to a nucleic acid construct that expresses an appropriate p73 coding sequence.

Any method of administration of p73 (e.g., as a protein or as a nucleic acid encoding the protein) is
30 appropriate as long as it results in increased levels of p73 protein within the target cell. The choice of method of administration will depend largely on the position of the target cells and the length of time the treatment is

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needed. Target cells may be removed from the patient and treated *ex vivo*, and then reintroduced to the patient. Additionally, the treatment may be used in cell cultures for experimental purposes. In a preferred embodiment, the target cells comprise E6-over-expressing carcinomas. In a more preferred embodiment, the target cells are papilloma-virus positive cancers. In a most preferred embodiment, the target cells are HPV-positive carcinomas of the uterine cervix.

10 The administration of p73 protein to target cells can be accomplished by exposing the target cell to p73 protein. When the target cell are tumor cells within an animal, it is preferred that the protein is administered in a protected form to increase their stability cells One strategy of accomplishing this is to use liposomes. Liposomes are water-filled vesicles composed of several phospholipids layers surrounding an aqueous core with an outer shell capable of providing direction to specific target cells. Typically liposomes are composed of some combination of phosphatidylcholine, cholesterol, phosphatidylglycerol or other glycolipids or phospholipids (Hudson and Black, 1993, American Pharmacy NS33(5):23-24). Insoluble polymers composed of polyethylene may also be used to form a protective layer around the protein, inhibiting degradation while traveling to the target cell (Hudson and Black, 1993, American Pharmacy NS33(5):23-24). Another way to deliver p73 protein to target cells is to couple the protein to a target cell-specific monoclonal antibody. This approach allows the protein to be specifically delivered to the target cell and minimizes toxic effects on non-target cells (Houston, 1993, Current Opinion in Biotechnology 4:739-744).

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In preferred embodiments, the p73 protein is administered to the target cell through the use of heterologous nucleic acids that will cause the protein to be synthesized within the target cell. These nucleic acids can be temporary residents in the target cell, such as expression plasmids, or they can be stably integrated into the genome of the target cell. Expression plasmids are particularly appropriate for experimental work with cell cultures, such as illustrated in Example 1. The construction of such plasmids and the transformation of target cells with them *in vitro* is well known to those of skill in the art of cell biology. Expression vectors suitable for p73 expression in mammalian cells are commercially available (Gene Therapy Systems, San Diego).

Naked DNA and plasmids may be delivered to the target cells by several known means. The naked DNA may be transferred directly into the genetic material of the cells (Wolff et al., 1990, Science 247:1465-1468), the p73-encoding DNA may be delivered in liposomes (Ledley, 1987, J. Pediatrics 110:1) or proteoliposomes that contain viral envelope receptor proteins (Nicolau et al, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:1068), or the p73-encoding DNA may be coupled to a polylysine-glycoprotein carrier complex.

For a longer lasting expression of p73 within target cells, viral vectors are preferred. A variety of viral vector may be used in this invention, included retroviral vectors such as the herpes simplex virus (U.S. Patent 5,288,641, incorporated herein by reference), Cytomegalovirus, murine leukemia virus (Blaese et al., 1995, Science 270:475-479) and similar as described by Miller (Miller, 1992, Curr. Top. Microbiol. Immunol. 158:1). Recombinant adeno-associated virus (AAV vectors)

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such as those described by U.S. Patent No. 5,139,941 (which is incorporated herein by reference) and recombinant adenoviral vectors (He et al., 1998, PNAS 95:2509-2514, incorporated by reference herein) are particularly preferred. Also contemplated are recombinant lentivirus vectors such as a recombinant Human Immunodeficiency Virus (U.S. Patent No. 5,885,805; Blaese et al., 1995, Science 270:475-479; Onodera et al., 1998, J. of Virology 72:1769-1774) and Feline Immunodeficiency Virus. Often these vectors have been designed so that they are replication-defective, and the techniques to prepare such vectors are well known in the art (Ghosh-Choudhury and Graham, 1987, Biochem. Biophys. Res. Comm. 147:964-973; McGrory, W. J. et al., 1988, Virology 163:614-617; Gluzman et al., 1982 in Eukaryotic Viral Vectors (Gluzman, Y., Ed.) pp. 187-192, Cold Spring Harbor Press, Cold Spring Harbor, N.Y). It is also contemplated that viral vectors that are replication competent may be used to improve the efficacy of the treatment of solid tumors (Wildner et al., 1999, Gene Ther. 6:57-62).

The recombinant vector of the invention comprises a nucleic acid construct comprising a sequence encoding a p73 protein operably linked to an appropriate promoter and other expression-regulatory sequences. For treatment of cancer cells, a strong constitutive promoter, such as a cytomegalovirus promoter, a viral LTR, RSV or SV40 promoter, is preferred. In a preferred embodiment, a cytomegalovirus promoter is used. Additionally, promoters associated with genes that are expressed at high levels in mammalian cells, such as elongation factor-1 and actin, are also contemplated. It is particularly advantageous to use a viral-specific and

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-regulated promoter to direct expression specifically in affected cancer cells. In a particularly preferred embodiment, the HPV-E6 promoter is used.

In a particularly preferred embodiment, a
5 recombinant adenoviral vector is used to deliver the p73-expressing construct to the target cell. The use of adenoviral vectors for gene therapy is well known in the art (El-Deiry et al., 1993, Cell 75:817; Blogosklonny and El-Deiry, 1996, Int. J. Cancer 67:386-395; Prabhu et al.,
10 1996, Clin Cancer Res. 2:1221-1230; Zeng et al., 1997, Int. J. Oncol. 11:221-226; Mitchell and El-Deiry, 1999, Cell Growth and Diff. 10:223-230; Meng et al., 1998, Clin. Cancer Res. 4:251-259; Blagosklonny and El-Deiry, 1998, Int. J. Cancer 75:933-940). In particular, an
15 adenovirus vector has been used successfully to deliver p53 to target cells to treat lung cancer in human patients (Roth et al., 1996, Nature Med. 2:974 incorporated herein by reference; and U.S. Patent 5,747,469 incorporated herein by reference). It is
20 contemplated that these protocols with simple variation that will be well known to those in the art can be used to administer the p73 protein to target cells in the invention. In a most preferred embodiment, therapeutically effective amounts of the viral vector are
25 delivered to the cancers by direct injection.

The interchangeability of p53 and p73 in these methods arises from the high degree of similarity that these proteins have, both in structure and function. p53 and p73 have significant amino acid sequence similarities
30 (Kaghad et al, 1997, Cell 90:809-818, incorporated by reference herein), particularly in the most conserved regions of p53: the transactivation, DNA binding and p53 oligomerization domains. A sequence similar to the MDM2-

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binding domain is also present in p73. The residues in p53 often found mutated in tumors and shown to be required for DNA recognition are conserved and occupy identical positions in p73. The C-terminal domain of p73 α shows homology to invertebrate p53 homologs. Finally the intron-exon organization of p73 is very similar to p53.

p53 and p73 are also functionally similar. Both display homotypic interactions, and p53 and p73 β display significant mutual interactions (Kaghad et al, 1997, Cell 90:809-818). Both are inhibited by adenovirus E4ORF6 (Higashino et al., 1998, PNAS 95:15683-15687) and the MDM2 oncoprotein (Zeng et al., 1999, Mol. Cell. Biol. 19:327-3266; Dobbelstein et al., 1999, Oncogene 18:2101-2106). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells in a manner similar to p53 (Di Como et al., 1999, Mol. Cell. Biol. 19:1438-1449). p73 regulates p53 target genes when p73 is over-expressed in cells (Zhu et al., 1998, Cancer Research 58:5061-5065; Jost et al., 1997, Nature 389:181-184). Finally, as a result of activation of p53-responsive genes, p73 can inhibit cell growth and induce apoptosis in a manner similar to p53.

Also provided with the invention are pharmaceutical compositions that can be used to treat mammalian cells with p73 *in vitro*, *in vivo* and *ex vivo*. The compositions comprise either p73 protein or nucleic acids encoding p73 protein. The pharmaceutical compositions of the invention are formulated in an appropriate "biologically acceptable medium". As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media and the like which may be appropriate for the desired route of administration of

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the pharmaceutical preparation, as exemplified in the preceding paragraph. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is
5 incompatible with the nucleic acid molecules or proteins to be administered, its use in the pharmaceutical preparation is contemplated.

The pharmaceutical preparation is formulated in dosage unit form for ease of administration and
10 uniformity of dosage. Dosage unit form, as used herein, refers to a physically discrete unit of the pharmaceutical preparation appropriate for the patient undergoing treatment. Each dosage should contain a quantity of active ingredient calculated to produce the
15 desired effect in association with the selected pharmaceutical carrier. Procedures for determining the appropriate dosage unit are well known to those skilled in the art.

The pharmaceutical composition also can include
20 various other components as additives or adjuncts. Exemplary pharmaceutically acceptable components or adjuncts which are employed in relevant circumstances include antioxidants, free radical scavenging agents, peptides, growth factors, antibiotics, bacteriostatic
25 agents, immunosuppressives, anticoagulants, buffering agents, anti-inflammatory agents, anti-pyretics, time release binders, anaesthetics, steroids and corticosteroids. Such components can provide additional therapeutic benefit, act to effect the therapeutic action
30 of the pharmaceutical composition, or act towards preventing any potential side effects which may be posed as a result of administration of the pharmaceutical composition. In certain circumstances, the p73 protein

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or nucleic acid molecule can be employed as part of a pharmaceutical composition with other compounds (e.g., chemotherapeutic agents) intended to prevent or treat cancer or a related disorder.

5 The manner in which the pharmaceutical preparations are administered can vary. They can be administered by inhalation (e.g., in the form of an aerosol either nasally or using delivery articles of the type set forth in U.S. Patent No. 4,922,901 to Brooks et al.); topically (e.g., in lotion form or as a suppository); orally (e.g., in liquid form within a solvent such as an aqueous or non-aqueous liquid, or within a solid carrier); intravenously (e.g., within a dextrose or saline solution); as an infusion or injection (e.g., as a suspension or as an emulsion in a pharmaceutically acceptable liquid or mixture of liquids); intrathecally; intracerebro-ventricularly; or transdermally (e.g., using a transdermal patch). Exemplary methods for administering such compounds will be apparent to the skilled artisan. The administration of the pharmaceutical compositions of the present invention can be intermittent, or at a gradual, continuous, constant or controlled rate to a warm-blooded animal, (e.g., a mammal such as a mouse, rat, cat, rabbit, dog, pig, cow, or monkey); but advantageously is preferably administered to a human being. In addition, the time interval between administrations can vary. Administration preferably is such that the active ingredients of the pharmaceutical formulation contact the target cells, whether within or outside the body of a mammalian subject.

The appropriate dose of the compound is that amount effective to result in increased levels of p73

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protein within the target cell. By "effective amount", "therapeutic amount" or "effective dose" is meant that amount sufficient to elicit the desired pharmacological or therapeutic effects, thus resulting in effective prevention or treatment of the disorder. Prevention of the disorder is manifested by delaying the onset of the symptoms of the disorder. Treatment of the disorder is manifested by a decrease in the symptoms associated with the disorder or an amelioration of the recurrence of the symptoms of the disorder.

The effective dose can vary, depending upon factors such as the condition of the patient, the severity of the symptoms of the disorder, and the manner in which the pharmaceutical composition is administered. The effective dose of compounds will of course differ from patient to patient but in general includes amounts starting where target cell growth is halted to where the target cell is killed. Dosages contemplated for use with the retroviral vector embodiment of the invention are those suggested in U.S. Patent 5,747,469 (incorporated herein by reference). One of ordinary skill in the art will know how to determine such doses without undue experimentation.

Kits with the components necessary to treat E6-over-expressing target cells by the method of the invention are also provided. In a preferred embodiment, the kit contains therapeutically effective amounts of the pharmaceutical preparation of the invention in a container. The pharmaceutical preparation in the kit may be comprised of p73 protein or a DNA construct encoding p73, preferably inserted into a vector for transforming cells. The p73 protein or p73 encoding viral vector may be in the form of a pharmaceutically acceptable sterile

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solution such as sterile saline, dextrose solution or buffered solution. Alternatively, the p73 protein or p73 encoding viral vector can be lyophilized or desiccated. In this instance the kit may optionally further comprise
5 a container of a pharmaceutically acceptable solution, (e.g., saline, dextrose solution, etc.), preferably sterile, to reconstitute the pharmaceutical preparation to form a solution for injection purposes. Optionally, instructions may be included in the kit. The kit may
10 additionally comprise pharmaceutical preparations in containers for other therapies related to cancer treatment, such as chemotherapy.

The following example is provided to describe
15 the invention in greater detail. It is intended to illustrate, not to limit, the invention.

EXAMPLE 1

20 p73 α and p73 β Suppress Growth and Induce Apoptosis in Human Papilloma Virus E6-Expressing Cancer Cells

Materials and Methods

Plasmids. The mammalian expression vector pCMV-neo-Bam (Baker et al., 1990, Science 249: 912-915)
25 and the wild-type p53 expression vector SN3 (Baker et al., 1990, Science 249: 912-915) were obtained from Bert Vogelstein (Johns Hopkins University). Wild-type and mutant p73 α and p73 β plasmids (Jost et al., 1997, Nature 389: 191-194; incorporated herein by reference) were
30 obtained from William G. Kaelin, Jr. (Dana Farber Cancer Institute). The HPV-E6 expression plasmid (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229; incorporated herein by reference) was obtained from Kathleen Cho (Johns Hopkins University).

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Cell culture and transfection conditions. The mutant p53-expressing human colon adenocarcinoma cell line SW480 was maintained in culture as previously described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). The mutant p53-expressing human glioma cell lines U373 and H80 were obtained from Peter C. Phillips (The Children's Hospital of Philadelphia) and the wild-type p53-expressing human non-small cell lung cancer cell line H460 was obtained from Stephen B. Baylin (Johns Hopkins University). Mutant p53-expressing SKBr3 cells were obtained from American Type Culture Collection (Rockville, MD). SW480 cells were transfected using Lipofectin (BRL) as previously described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). At 20 hrs. following transfection, cells were harvested and protein lysates electrophoresed through 10% polyacrylamide gels and immunoblotted as previously described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). Analysis of p53 expression was performed using the anti-human p53 monoclonal antibody pAb1801 (Ab2; Oncogene Science). For detection of exogenous p73 protein expression, the anti-HA antibody was used as previously described (Jost et al., 1997, Nature 389: 191-194).

Adenovirus infections. The Ad-LacZ reagent (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229) was obtained from Bert Vogelstein. The HPV type 16 E6-expressing replication deficient adenovirus was prepared and titered as previously described (Satyamoorthy et al., 1997, Cancer Res. 57: 1873-1876; Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). Briefly, the CMV promoter-driven HPV type 16 E6 cDNA was inserted into an E3-deleted adenovirus by homologous recombination to generate E1 and E3 deleted replication

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defective Ad-E6 adenovirus (Satyamoorthy et al., 1997, Cancer Res. 57: 1873-1876; incorporated herein by reference). The cloned HPV-E6 DNA sequence was verified and expression of HPV-E6 was verified by Northern blotting of total RNA derived from Ad-E6 versus Ad-LacZ infected cells. Cells were infected using an MOI of 50 as previously described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). Infection of SW480 cells using Ad-LacZ at an MOI of 50 followed by X-gal staining revealed greater than 99% infectivity.

Colony suppression assays. Transfections were carried out as described above except that the tumor suppressive (p53 or p73) or control (pCMV-neo-Bam) plasmid represented 80% of the total DNA and the degrading (pCMV-E6) or control (pCMV-neo-Bam) represented the remaining 20% of the total DNA. At 24 hrs following transfection, G418 selection was begun using 1 mg/ml as a final concentration. Selection was continued for 7-12 days and colony growth was analyzed as previously described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229).

TUNEL assays. At 48 hrs following transfection, cells were formalin fixed and the extent of apoptosis was assessed by nicked-end labeling using the Apotag kit (Oncor) followed by analysis using fluorescence microscopy.

Results

HPV-E6 targets both wild-type and mutant p53 protein for degradation. Using an E6-expressing adenovirus (Ad-E6) a panel of human cancer cells derived from different tissues and containing either endogenous wild-type or mutant p53 were infected (Fig. 1). As

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compared with Ad-LacZ infected cells, E6-expressing cells expressed substantially reduced levels of either wild-type or mutant p53 protein (Fig. 1, compare even to odd lanes). Thus the HPV-E6 protein targets both
5 wild-type and mutant p53 for degradation. E6 does not target the cell cycle regulatory proteins pRb, p21, cyclin E or p27 for degradation (Fig. 1 lower panels). The phosphorylation state of Rb in Ad-E6 was not altered as compared to Ad-LacZ infected cells (Fig. 1 lower
10 panels), regardless of the p53 status of the cells.

p73 is resistant to HPV E6-dependent proteolysis. Because p53 is degraded in HPV-E6 expressing cancer cells, such cells are not ideally suited for gene replacement therapy (Prabhu et al., 1996,
15 Clin. Cancer Res. 2: 1221-1229). In HPV E6-expressing cancer cells, p53 is degraded while exogenous p73 is resistant to E6-targeting to the proteasome (Fig. 2). The resistance of p73 to E6-dependent proteolysis was observed with p73 α , p73 α m, p73 β , or p73 β m. This
20 observation suggested that p73 is a candidate for gene replacement in E6-expressing cancer cells.

p73 β induces apoptosis and suppresses growth in HPV E6-expressing human cancer cells. p73 β was previously found to be a potent activator of
25 p53-dependent gene expression (Kaghad et al., 1997, Cell 90: 809-819; Jost et al., 1997, Nature 389: 191-194). p73 β in colony suppression assays in the absence or presence of E6-expression. Whereas p53 failed to inhibit the growth of E6-expressing cancer cells, p73 β was found
30 to be a potent growth suppressor. Transfection studies revealed that p73 α was a less potent suppressor of growth of SW480 cancer cells either in the absence or presence of HPV-E6.

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This could not be explained by dominant negative inhibition of p73 α by the endogenous p53 mutant in SW480 cells because it was previously shown that p73 α shows negligible interaction with p53 (Kaghad et al., 1997, Cell 90: 809-819).

p73 β has been previously shown to be an inducer of apoptosis, similar to p53 (Jost et al., 1997, Nature 389: 191-194). Whereas p53-dependent apoptosis was inhibited in E6-expressing cells, p73 β was still capable of inducing apoptosis similar to what is observed in the absence of E6. Therefore the colony suppression phenotype observed following p53 or p73 β expression in the presence or absence of E6 can be explained by their ability to induce apoptosis under these conditions. These results suggest that the p73 β -dependent suppression of growth of HPV E6-expressing cancer cells occurs through an apoptotic mechanism.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.

What is claimed:

1. A method of inducing apoptosis in an E6-expressing cell, comprising administering to the cell an
5 amount of p73 protein effective to induce the apoptosis.

2. The method of claim 1 wherein the p53 protein is administered as a DNA construct comprising an expressible sequence that encodes the p73 protein.
10

3. The method of claim 2, wherein the DNA construct is operably inserted into a viral vector.

4. The method of claim 3, wherein the viral
15 vector is selected from the group consisting of adenoviral vectors, HIV vectors, FIV vectors, herpes viral vectors, adeno-associated vectors and cytomegaloviral vectors.

20 5. The method of claim 1, wherein the cell is a cancerous cell.

6. The method of claim 1, wherein the cell is infected with Human papilloma virus.
25

7. The method of claim 1, wherein the cell is a cultured cell.

8. The method of claim 1, wherein the cell is
30 obtained from the body of a living organism, the administering is performed *ex vivo*, and the cell is returned to the living organism.

9. The method of claim 1, wherein the cell is disposed within a living organism and the administering is performed *in vivo*.

5 10. The method of claim 1, wherein the cell is obtained from a species selected from the group consisting of *Homo sapiens*, *Mus musculus* and *Chlorocebus aethiops*.

10 11. The method of claim 1, wherein the p73 protein is p73 α or p73 β .

15 12. The method of claim 11, wherein the protein comprises a sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

20 13. The method of claim 2, wherein the DNA construct comprises more than 50 nucleotides of SEQ ID NO:3.

25 14. A pharmaceutical preparation for treatment of cancers associated with E6 over-expression, comprising p73 protein associated with a delivery vehicle for delivering the preparation to cancer cells.

30 15. The pharmaceutical preparation of claim 14, wherein the p73 protein is p73 α or p73 β .

35 16. The pharmaceutical preparation of claim 15, wherein the protein comprises a sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

17. The pharmaceutical preparation of claim 14, which further comprises at least one additional active ingredient for treatment of cancer.

5 18. A kit comprising a container containing one or more dosage units of the pharmaceutical composition of claim 14.

10 19. The kit of claim 18, which further comprises at least one additional pharmaceutical agent for treatment of cancer.

15 20. A pharmaceutical preparation for treatment of cancers associated with E6 over-expression, comprising an expressible DNA construct encoding p73, associated with a delivery vehicle for delivering the preparation to cancer cells.

20 21. The pharmaceutical preparation of claim 20, wherein the DNA construct is operably inserted into a vector for transforming cells.

25 22. The pharmaceutical preparation of claim 21, wherein the vector is a viral vector selected from the group consisting of adenoviral vectors, HIV vectors, FIV vectors, herpes viral vectors, adeno-associated vectors and cytomegaloviral vectors.

30 23. A kit comprising a container containing one or more dosage units of the pharmaceutical composition of claim 20.

24. The kit of claim 23, which further comprises at least one additional pharmaceutical agent for treatment of cancer.

5 25. An apoptotic, E6-expressing transgenic cell comprising a heterologous, expressible DNA construct encoding p73.

10 26. The cell of claim 25, obtained from a cultured cell line.

 27. The cell of claim 25, disposed within a living organism.

15 28. The cell of claim 25, from a species selected from the group consisting of *Homo sapiens*, *Mus musculus* and *Chlorocebus aethiops*.

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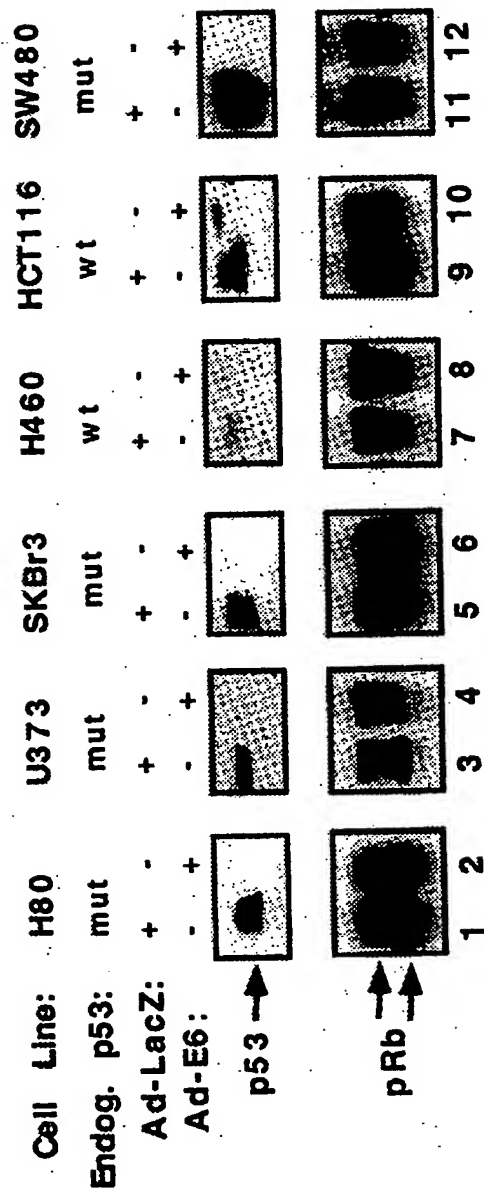


Figure 1

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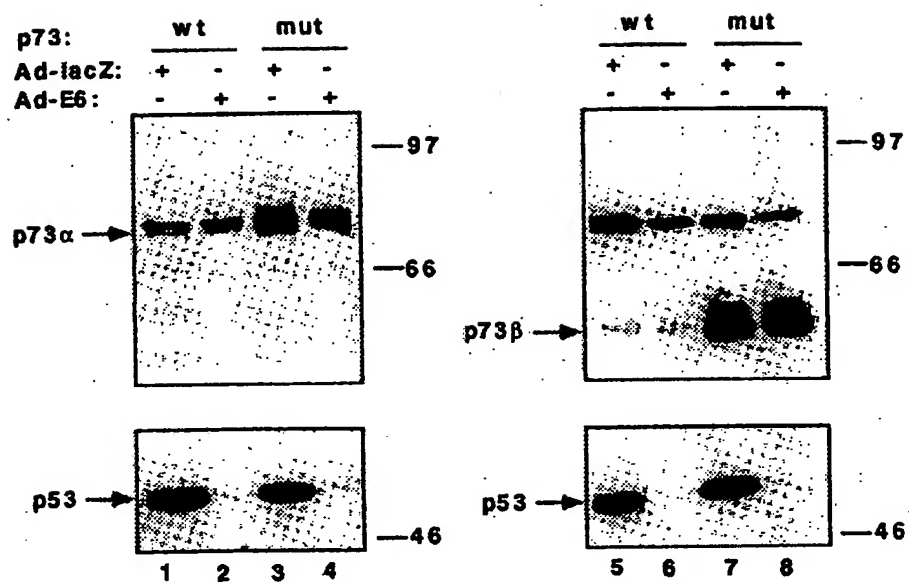


Figure 2

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| Met | Ala | Gln | Ser | Thr | Ala | Thr | Ser | Pro | Asp | Gly | Gly | Thr | Thr | Phe | Glu | 1 | 5 | 10 | 15 |
| His | Leu | Trp | Ser | Ser | Leu | Glu | Pro | Asp | Ser | Thr | Tyr | Phe | Asp | Leu | Pro | 20 | 25 | 30 | |
| Gln | Ser | Ser | Arg | Gly | Asn | Asn | Glu | Val | Val | Gly | Gly | Thr | Asp | Ser | Ser | 35 | 40 | 45 | |
| Met | Asp | Val | Phe | His | Leu | Glu | Gly | Met | Thr | Thr | Ser | Val | Met | Ala | Gln | 50 | 55 | 60 | |
| Phe | Asn | Leu | Leu | Ser | Ser | Thr | Met | Asp | Gln | Met | Ser | Ser | Arg | Ala | Ala | 65 | 70 | 75 | 80 |
| Ser | Ala | Ser | Pro | Tyr | Thr | Pro | Glu | His | Ala | Ala | Ser | Val | Pro | Thr | His | 85 | 90 | 95 | |
| Ser | Pro | Tyr | Ala | Gln | Pro | Ser | Ser | Thr | Phe | Asp | Thr | Met | Ser | Pro | Ala | 100 | 105 | 110 | |
| Pro | Val | Ile | Pro | Ser | Asn | Thr | Asp | Tyr | Pro | Gly | Pro | His | His | Phe | Glu | 115 | 120 | 125 | |
| Val | Thr | Phe | Gln | Gln | Ser | Ser | Thr | Ala | Lys | Ser | Ala | Thr | Trp | Thr | Tyr | 130 | 135 | 140 | |
| Ser | Pro | Leu | Leu | Lys | Lys | Leu | Tyr | Cys | Gln | Ile | Ala | Lys | Thr | Cys | Pro | 145 | 150 | 155 | 160 |
| Ile | Gln | Ile | Lys | Val | Ser | Thr | Pro | Pro | Pro | Pro | Gly | Thr | Ala | Ile | Arg | 165 | 170 | 175 | |
| Ala | Met | Pro | Val | Tyr | Lys | Lys | Ala | Glu | His | Val | Thr | Asp | Val | Val | Lys | 180 | 185 | 190 | |
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2 / 4

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 Arg Cys Pro Asn His Glu Leu Gly Arg Asp Phe Asn Glu Gly Gln Ser
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 Ala Pro Ala Ser His Leu Ile Arg Val Glu Gly Asn Asn Leu Ser Gln
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 40 Tyr Val Asp Asp Pro Val Thr Gly Arg Gln Ser Val Val Val Pro Tyr
 225 230 235 240
 Glu Pro Pro Gln Val Gly Thr Glu Phe Thr Thr Ile Leu Tyr Asn Phe
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 45 Met Cys Asn Ser Ser Cys Val Gly Gly Met Asn Arg Arg Pro Ile Leu
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 Ile Ile Ile Thr Leu Glu Met Arg Asp Gly Gln Val Leu Gly Arg Arg
 275 280 285
 Ser Phe Glu Gly Arg Ile Cys Ala Cys Pro Gly Arg Asp Arg Lys Ala
 290 295 300
 50 Asp Glu Asp His Tyr Arg Glu Gln Gln Ala Leu Asn Glu Ser Ser Ala
 305 310 315 320
 Lys Asn Gly Ala Ala Ser Lys Arg Ala Phe Lys Gln Ser Pro Pro Ala
 325 330 335
 55 Val Pro Ala Leu Gly Ala Gly Val Lys Lys Arg Arg His Gly Asp Glu
 340 345 350
 Asp Thr Tyr Tyr Leu Gln Val Arg Gly Arg Glu Asn Phe Glu Ile Leu
 355 360 365
 60 Met Lys Leu Lys Glu Ser Leu Glu Leu Met Glu Leu Val Pro Gln Pro
 370 375 380
 Leu Val Asp Ser Tyr Arg Gln Gln Gln Gln Leu Leu Gln Arg Pro Ser

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	Gly Pro Gly Met Leu Asn Asn His Gly His Ala Val Pro Ala Asn Gly												
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